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Establishment of Xenografts and Cell Lines From Well-differentiated Human Thyroid Carcinoma

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Background: Our aim was to establish Chinese human thyroid cancer cell lines and to provide the material for thyroid cancer research.

Methods: We collected thyroid cancer tissues from 93 patients with thyroid cancer for developing the primary culture. Thyroid cancer tissues were verified by frozen sections during the operations, then subjected to primary cell culture. During the first several passages, fibroblasts were removed by selective attachment.

Results: From the 93 cancer tissues used, two follicular cancer tissues from the metastatic area (CGTH W-1, CGTH W-2) and one papillary thyroid cancer tissue (CGTH W-3) could be passed over 50 times. Cellular transformation with loss of contact inhibition occurred during passages 5–8. Electron microscopic studies of the CGTH W-1 cell line showed the presence of an abundance of mitochondria and Golgi complex. Presence of microvilli with interdigitations between neighboring cells were found in CGTH W-2 and CGTH W-3 cell lines. Severe combined immunodeficient (SCID) mice were used to determine whether these cells were tumorigenic. Two months after transplantation of 1×10^7 CGTH W-1, 2, and 3 cells into SCID mice, subcutaneous tumors ~ 2–2.5 cm in size were clearly visible. After affinity cross-linking, an IGF-I with insulin-like growth factor binding proteins complex corresponding to a molecular size of 41 kDa was observed in culture media collected from CGTH W-1, SW 579, and RO82 W-1 cells.

Conclusions: Three thyroid cancer cell lines were established, which may provide material for future thyroid cancer research.

KEY WORDS: **follicular thyroid carcinoma, papillary thyroid carcinoma, immortalization, primary culture**

INTRODUCTION

Tissue culture was devised to study the behavior of animal cells at the beginning of this century [1,2]. Human tumor tissue was demonstrated to give rise to continuous cell lines in 1952 [3]. Using the culture system in the experiments, we were able to control the environment and to characterize and obtain homogeneous samples with economic advantages. Since most thyroid cancers were well differentiated and slow growing, human follicular thyroid carcinoma cell line RO82 W-1 was not characterized until 1989 by Estour et al. [4]. This cell line was established from the metastatic tissue of a follicular carci-

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noma from a female patient. The cells retain the capacity to secrete thyroglobulin (Tg) in vitro but are unable to concentrate ^{131}I. This thyroid cancer cell line containing retinoic acid and estrogen receptors has been used in many studies [5–7]. In the same year, two human medullary thyroid carcinoma cell lines, which displayed the ability to secrete calcitonin, were established and characterized [8]. We have begun to work on the primary culture with human thyroid cancer tissues in 1991. Our aims were to establish Chinese human thyroid cancer lines so as to provide our materials for thyroid cancer research.

MATERIALS AND METHODS

During the last 3 years, we collected tissues from 93 patients with thyroid cancer for initiating primary culture. Most of these patients underwent nearly total thyroidectomy in Chang Gung Medical Center. Among these tissues, there were 70 papillary thyroid carcinomas, 14 follicular thyroid carcinomas, 7 anaplastic thyroid carcinomas, and 2 medullary thyroid carcinomas.

All thyroid cancer tissues were verified by frozen sections during operations and were kept in an RPMI 1640 medium at room temperature. The tissues immediately were sent to the tissue culture room for processing. They were minced into < 1 mm^3 in size and washed three times in RPMI 1640 medium after careful removal of connective tissue and fat. After centrifugation at 700 rpm for 5 min, the pellet was resuspended in RPMI 1640 medium containing 0.2 mg/mL collagenase I and IV (4:1) without fetal calf serum (FCS). Then, tissues were incubated at 37°C with shaking overnight. The debris was macerated through a screen. The cells were harvested by centrifugation at 700 rpm for 5 min and resuspended in RPMI 1640 medium containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, 10 μg/mL insulin, 5×10^{-6} M hydrocortisone, and 25 ng/mL epidermal growth factor. The cells were placed in Petri dishes precoated with collagen and incubated at 37°C in 5% CO_2 incubator. The medium was changed every 48–72 hr after the epithelial cells were attached. When the cell culture reached confluence, the cells were washed once with RPMI 1640 medium. Then trypsin (0.5 g/L, dil 1:250) and EDTA (0.2 g/L) (Irvine Scientific, Irvine, CA) were added to detach the cells. The detached cells were collected and resuspended in 10 ml of RPMI 1640 medium. The viability of the cells was determined by trypan blue dye exclusion. The culture was then propagated and passed on.

During the first few passages, fibroblasts were removed by selective attachment. Growth factors were removed after the 10th passage. For determining the serum requirement of cultured cells, the cells were plated in RPMI 1640 medium supplemented with FCS at concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0%. The number of cells were determined at 36 and 96 hr. Ultrastructural studies were performed by transmission electron microscopy. After harvesting cells by a policeman from the culture flask, the cells were fixed with 2.5% v/v of glutaraldehyde in RPMI 1640 and postfixed with 2% osmium tetroxide for electron microscopic observation.

Detection of insulin-like growth factor binding proteins (IGFBP) by affinity cross-linking was performed in RO82 W-1 and SW 579 and CGTH W-1 cell lines [9]. The well-differentiated human thyroid follicular cancer line, RO82 W-1, was obtained from Dr. Andre Van Herle (Los Angeles, CA) [4]. The anaplastic thyroid cancer cell line, SW 579, was purchased from ATCC. Cells were cultured in a 75 cm^2 flask with RPMI 1640 medium containing 10% FCS. After the cells grew to confluence, FCS-free RPMI 1640 medium was used for further culture. After a 48-hr culture period, the conditioned medium was collected for detection of IGFBPs by SDS-PAGE after affinity cross-linking. Twenty-five μl of the conditioned medium was incubated with ^{125}I-labeled IGF-I (75,000 cpm) in the absence or presence of unlabeled IGF-I (500 ng/tube) at 4°C for 16 hr (final volume, 95 μl in 0.05 M PBS, pH 7.4). RPMI 1640 medium was used as the control. After incubation, 5 μl of 0.02 M disuccinimidyl suberate (Pierce Chemical, Rockford, IL) freshly dissolved in dimethyl sulphoxide was added and followed by further incubation at room temperature for 30 min. At the end of incubation, 100 μl sample buffer (0.625 M Tris, 2% SDS, 10% glycerol, 0.7 M β-mercaptoethanol and 0.01% bromophenol blue, pH 6.8) was added to each sample. The samples were boiled for 5 min and loaded on to discontinuous SDS-PAGE (12.5% running gel and 3% stacking gel). Following staining by Coomassie blue R, the gels were dried and examined by autoradiography.

In the immunocytochemical staining, the cells (5×10^7) were trypsinized, washed three times with RPMI 1640 medium. The cell pellet was fixed in 10% formalin and embedded in paraffin. Trypsin-treated sections (5 mm thick) were immunostained by the avidin-biotin peroxidase complex (ABC) method. They were incubated with 5% nonfat milk-blocking solution at room temperature for 30 min. After washing three times with PBS, the cells were incubated with mouse antihuman Tg monoclonal antibody (Chemicon, Temecula, CA), mouse antihuman fibroblast monoclonal antibody (Dako, Copenhagen, Denmark), and kept overnight in a moist chamber. At the end of incubation with the primary antibody, the cells were incubated with biotinylated goat antimouse secondary antibodies (Vector, Burlingame, CA) for 60 min. After washing three times with PBS, the cells were reacted with Vectastain elite ABC reagent (Vector) for 45 min. The cells were then treated with 0.05% diaminobenzidine-hydrogen peroxide-peroxidase solution and counterstained with methyl green (Sigma, St. Louis, MO). As a control, fibroblast from the thyroid cancer tissue was used after several generations of the primary culture.

The fibroblast was proved by immunocytochemical staining using monoclonal mouse antihuman fibroblast antibody (Dako, Copenhagen, Denmark).

To determine whether these cells were tumorigenic, 1×10^7 of the passages 20–25 suspended in RPMI 1640 medium were injected subcutaneously into severe combined immunodeficient (SCID) mice (9–12 weeks old). The animals were kept in a semisterile environment. One month later, the SCID mice were killed and the tumors were removed for pathological examination. Another three SCID mice, injected with the 1×10^7 of fibroblasts from the papillary thyroid cancer tissue, were used as controls.

RESULTS

Forty-eight hours after plating in Petri dishes, most of the isolated thyroid cancer cells began to attach on the dishes. The papillary thyroid cancer cells look longer than the follicular thyroid cancer cells (Fig. 1). The growth rates of the cancer cells in vitro seemed to vary markedly; in addition, immortalization of the cancer cells did not correlate with the proliferative rate of the cancer cells in the beginning several passages. Figure 2A demonstrates the follicular thyroid cancer cells from a 60-yr-old female patient, which grew into monolayer cells 48 hrs after plating. But after the first passage (Fig. 2 B), the cells turned more flat. No more attachment of the cells was found after the second passage. Figure 2C illustrates the cancer cells removed from the pulmonary metastases of the same patient; these cells revealed quite different morphology from the primary cancer cells. during the first several passages, fibroblast overgrowth and detachment of the cancer cells were the major problems. After 5–8 passages, most of the epitheloid cells turned senescence.

Among the 93 cancer tissues tested, two follicular cancer tissues from the metastatic area (CGTH W-1, CGTH W-2) and one papillary thyroid cancer tissue (CGTH W-3) could be passed over 50 times. Cellular transformation with loss of contact inhibition (Fig. 3) occurred during passages 5–8. The CGTH W-1 cell line was established from a follicular thyroid carcinoma metastasizing to the sternum of a 70-yr-old Chinese female patient. The metastatic lesion in the sternum was verified as follicular thyroid cancer by the final pathological section. Well-differentiated thyroid follicular structure was found in the metastatic tissue (Fig. 4). The cancer cell line that could be maintained longest was from CGTH W-1. This cell line has been maintained for > 100 passages. The morphology of this did not change during this period, as determined by microscopic examination.

The CGTH W-2 cell line was established from the metastatic tissue to the skull of a 56-yr-old female follicular thyroid cancer patient. The patient had received the first operation of nearly total thyroidectomy and resection of metastatic tissues at the occipital skull 6 years earlier.

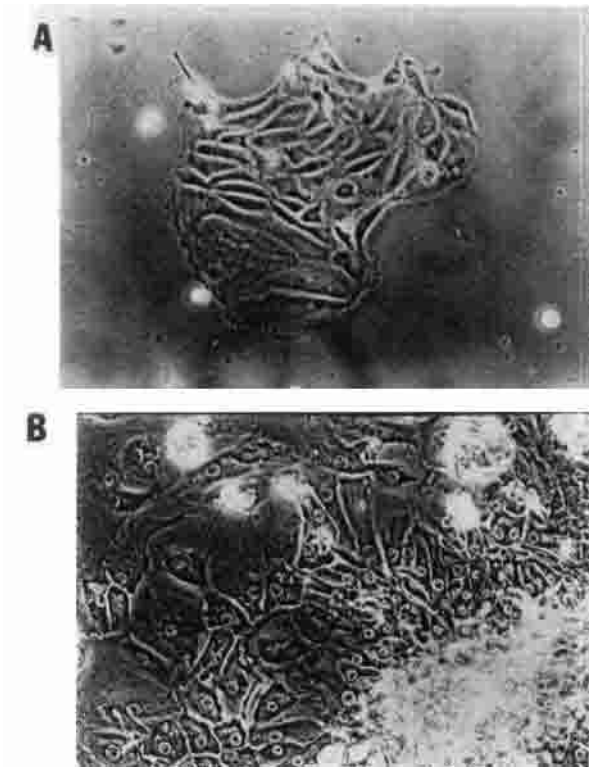

Fig. 1. **A:** Primary culture of a papillary thyroid cancer tissue. The tissue was removed from the papillary thyroid carcinoma of a 61-year-old female patient. At 72 hr after the plating, a cluster of epitheloid cells revealed a longer shape when compared with the tissue from follicular cancer. **B:** Primary culture of follicular cancer of a 49-yr-old male patient.

A second operation was done 1 year earlier to remove the left frontal skull metastasis. In vivo studies demonstrated that the metastatic cancer cells still retained the ability to trap iodine and to secrete Tg. These findings were confirmed by the postoperative positive ^{131}I scan and the high serum Tg level of 7500 ng/mL, respectively. The CGTH W-3 line was established from the papillary thyroid carcinoma tissue of a 31-year-old female patient. In vitro studies of the three cell lines during the first six passages, Tg could be detected.The concentrations ranged from 2.3 to 10 ng/mL in the conditioned culture medium in contrast to undetectable Tg concentration in the medium without culture (Table I). Tg levels were detected by a Tg kit (CIS bio international, France). The detection limit of the Tg kit was 0.5 ng/mL. Interassay coefficient of variation was 8% at Tg level of 4.9 ng/mL, 6.9% at 223.2 ng/mL, 5.1% at 312.9 ng/mL. However, after spontaneous transformation of the cells (i.e., piling up of the cells), Tg concentration could not be detected in the conditioned culture medium.

After the 10th passage, the removal of insulin, hydrocortisone, and epidermal growth factors did not affect cell growth, Figure 5 illustrates that the optimal condition

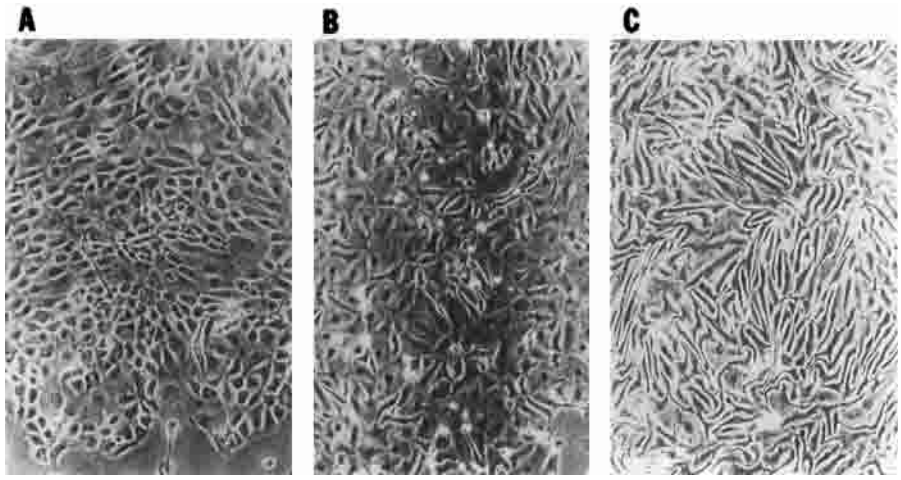

Fig. 2. **A:** Monolayer cell of follicular thyroid cancer tissue 48 hr after plating. **B:** After the cells were trypsinized; the cells turned flat in the secondary passage. **C:** Monolayer cells of the metastatic follicular thyroid cancer tissue from the lung of the same patient.

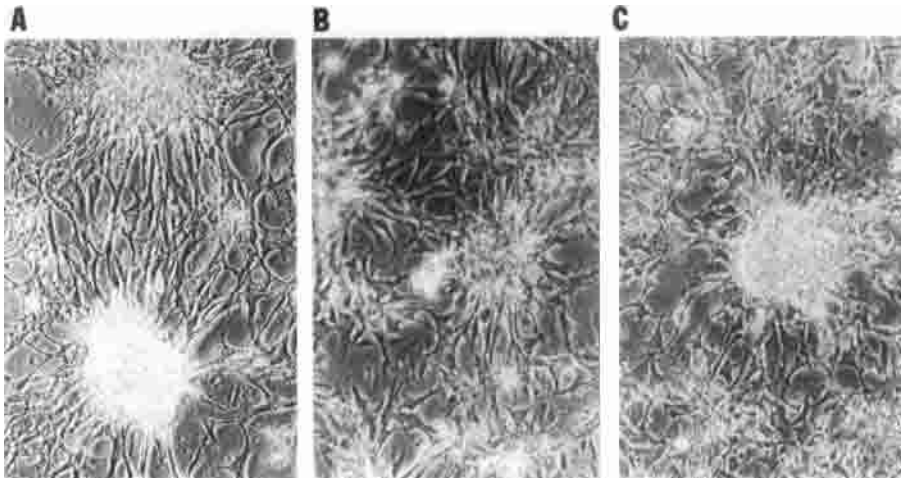

Fig. 3. Spontaneous transformations occurred during passages 5–8 of CGTH W-1 (**A**), W-2 (**B**), and W-3 (**C**) cell lines.

for cell growth was attained with a 10% FCS media. But even under a very low concentration of FCS, e.g., 0.5%, the cell can still grow well. The cell population doubling times evaluated by cell counting of the CGTH W-1 and CGTH W-2 cultured in the 10% FCS were about 18 and 20 hr, respectively.

Electron microscopic studies of the CGTH W-1 cell line showed the presence of an abundance of mitochondria and Golgi complex (Fig. 6). Presence of microvilli with interdigitations between neighboring cells was found in CGTH W-2 and CGTH W-3 cells lines. Under electron microscopic examination, no secretory granule or tight junction was found in these cell lines.

After affinity cross-linking, an IGF-I-IGFBP complex corresponding to a molecular size of 41 kDa was observed in culture media collected from CGTH W-1, SW 579, and RO82 W-1 cells (Fig. 7, lanes A, C, D). Non-specific binding of ^{125}I-labeled IGF-I cross-linked by disuccinimidyl suberate was assessed by adding unlabeled IGF-I to culture media during incubation (Fig. 7, lane B).

Two months after injecting 1×10^7 CGTH W-1, W-2, W-3 cells into SCID mice, subcutaneous tumors were 2–2.5 cm in size (Fig. 8). Four months after transplantation, the largest tumor had grown to 3 cm in size. Distant metastases to the infrahepatic area and the omentum were observed 3 months after the transplantation. In contrast, transplantation of fibroblasts from the human cancer tissues did not form tumors in the SCID mice even 4 months after the transplantation. All the SCID mice implanted with CGTH W-1, W-2, W-3 expired within 130 days after

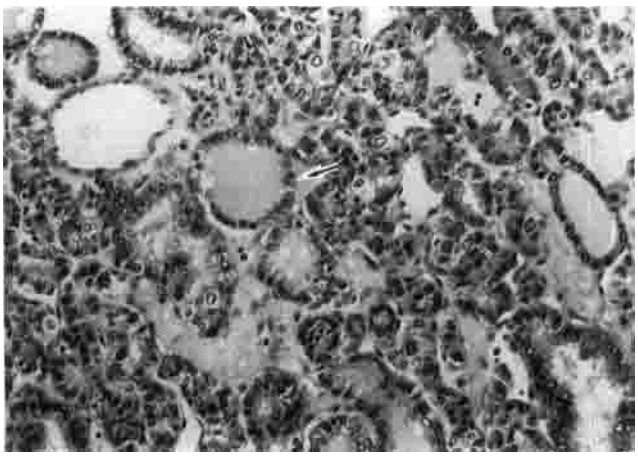

Fig. 4. Metastatic follicular thyroid cancer tissue removed from the sternum of the patient, which revealed thyroid follicular structure (arrow) (H & E, 200×).

TABLE I. Thyroglobulin Concentrations of the Conditioned Media from Different Passages of Various Thyroid Cancer Cell Lines

Cell lines	Passage	Thyroglobulin concentration (ng/dL)
CGTH W-1	1	5.6
CGTH W-2	1	10.0
CGTH W-3	1	8.3
CGTH W-1	2	6.3
CGTH W-2	3	8.6
CGTH W-3	4	2.3
CGTH W-1	8	0
CGTH W-2	10	0
CGTH W-3	9	0

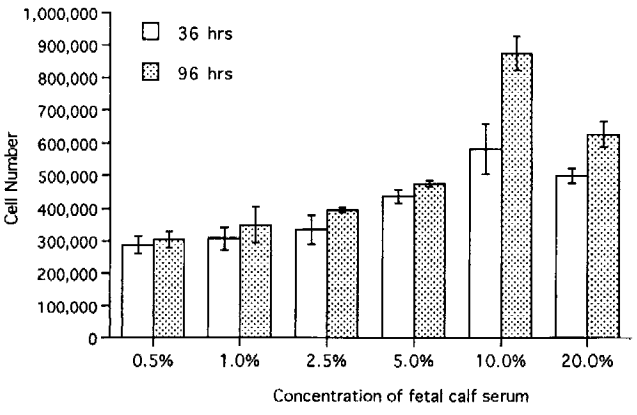

Fig. 5. Serum requirement of CGTH W-1 cells; 2.5×10^4 of the CGTH W-1 cells were plated in RPMI 1640 medium supplemented with various concentrations of fetal calf serum (FCS). The number of cells were counted at 36 hr and 96 hr.

the transplantation, The sections of the tumor tissues were examined after hemotoxylin and eosin staining. Part of the removed tissue was used for primary culture as described

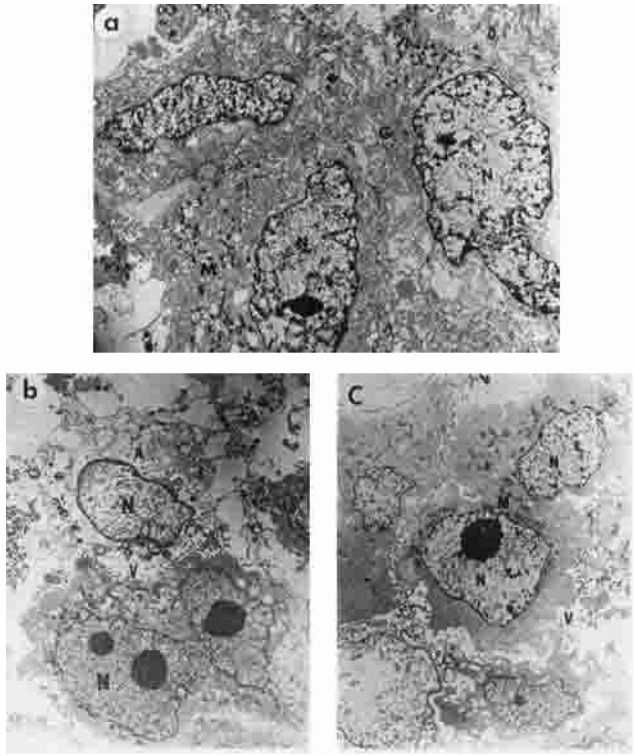

Fig. 6. **A:** An abundance of mitochondria (M) and Golgi complex (G) with nucleus (N) were observed in CGTH W-1 cell (3000×). Presence of microvilli with interdigitations between neighboring cells were found in CGTH W-2 **(B)** and CGTH W-3 **(C)** lines.

above, and the cells could be maintained and passed for >10 times.

Immunocytochemical staining with antihuman Tg monoclonal antibody revealed negative staining after six times of the passage of all three cell lines.

DISCUSSION

It is usually difficult to obtain enough specimen for the experiments involving human tissues. Cancer cell immortalization was a key step to ensure the source of the cells for the studies [10,11]. In the present study, most of the primary cultures from thyroid cancer became quiescent within three passages. Rapid fibroblast overgrowth was found if the epitheloid cells failed to form a monolayer within 2 weeks. Chemically defined medium with 0.5% bovine serum with other growth factors had been shown to inhibit fibroblast overgrowth [12], but under such conditions the human thyroid cancer cell could not proliferate unless the cells were transformed.

In our primary culture, most of the cells underwent apoptosis, defined as active cell death in the absence of immune surveillance [13]. In vivo, apoptosis has been demonstrated to be responsible for the normal elimination of cells with damaged DNA [14]. Although immortalization of the cell was loosely defined, the term was used to

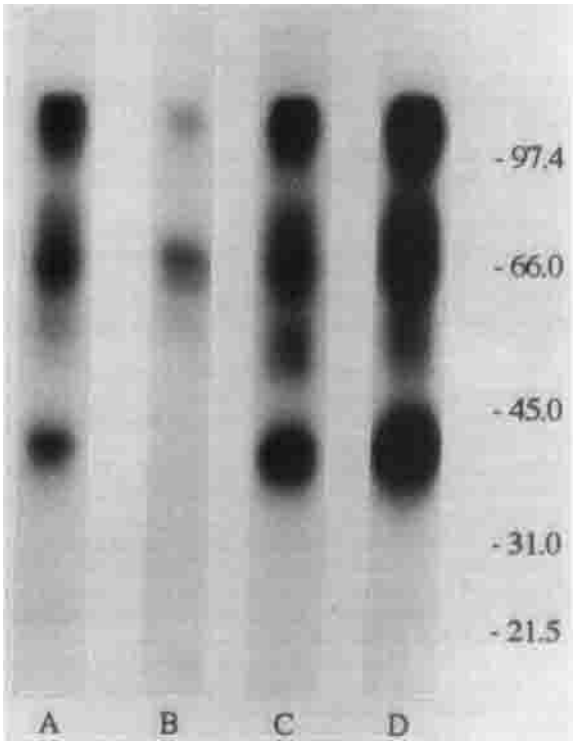

Fig. 7. Detection of IGFBPs by affinity cross-linking. An IGF-I-IGFBP complex corresponding to a molecular size of 41 kDa was observed in culture media collected from CGTH W-1, SW 579, and RO82 W-1 cells **(A, C, D).** Nonspecific binding of ^{125}I-labeled IGF-I cross-linked by disuccinimidyl suberate was assessed by adding unlabeled IGF-I to culture media during incubation **(B).**

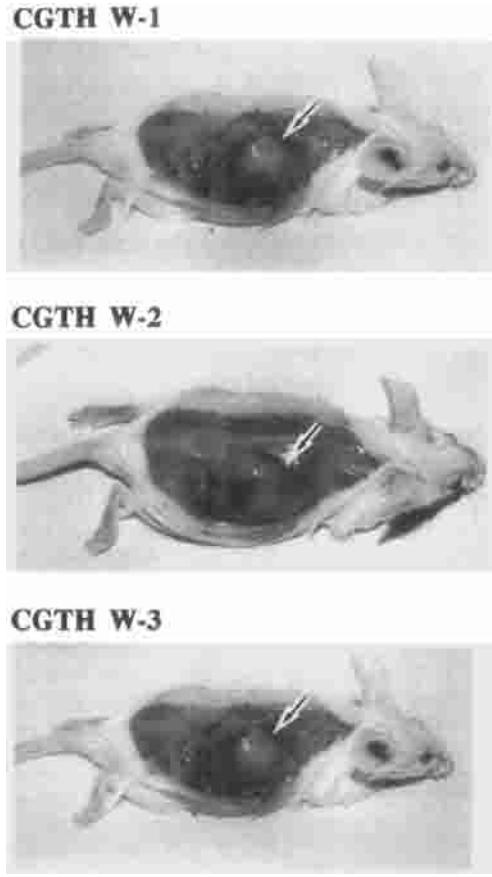

Fig. 8. Tumorigenicity studies of CGTH W-1, 2, and 3 cells. Two months after 1×10^7 CGTH W-1, 2, and 3 cells were injected into the subcutaneous tissue, a tumor of ~ 2 cm in diameter (arrow could be seen clearly.

denote an infinite lifespan. For immortalization of human cells, chemicals, physical agents, oncogenes, and DNA tumor viruses have been used as immortalization agents [12,15]. In our primary culture, we did not use these reagents or viruses. Spontaneous in vitro transformation of human cultured cells has been relatively rare [16,17]. Among our 93 human thyroid cancer tissues, spontaneous transformations occurred in three cancer tissues (3.2%). Using plasmid pSV3neo transfected into normal human thyrocytes, Whitley et al. [18] could immortalize a thyrotrophin-responsive cell line [15]. In our cell lines, the karyotype and the presence of IGF-1 receptor had been reported in our previous studies. IGF binding protein secretion was proved in CGTH W-1, RO82 W-1, and SW 579 cell lines, but [^{3}H] thymidine incorporation studies failed to prove that thyrotropin could enhance cellular proliferation in these cell lines.

In recent studies, SCID mice have been successfully used to preserve functioning human thyroid organoids [19]. The CGTH W-1, W-2, and W-3 cell lines grew very well after subcutaneous injection into SCID mice. All the SCID mice died of tumor metastases within 5 months. This SCID mouse model can provide an in vivo study system for human thyroid cancers. [20].

During the passages of these primary cultures, the cells lost their ability to attach to the substrate. The anchorage-dependent phenomenon has been related to apoptosis of the cells [21]. Although the cells were still alive in the suspension of the culture medium, no mitosis could be detected. Guadagno et al. [22] demonstrated that anchorage-dependent cells growing in suspension were arrested in the G1 phase. During the first several passages, most cancer cells in the primary culture progressively lost their ability to attach to the cultured dishes. Integrins have been mentioned to be responsible for the adhesion [21]; however, their role in cellular immortalization needs to be further investigated. All three cell lines established in this study maintained their ability to attach to petri dishes until now but have lost their ability of contact inhibition during the passages.

In conclusion, three human thyroid cancer cell lines were established from 93 thyroid carcinoma tissues in this study. These cell lines included two follicular and one papillary thyroid carcinoma. After transformation, these cell lines lost their ability to secrete Tg. The ability of IGF binding proteins secretion were proved in CGTH

W-1, RO82 W-1, and SW 579 cell lines, which may play a role in the regulation of IGF-1 actions. The model established in this study of the cancer cell growing in SCID mice can be provided for in vivo studies of human thyroid cancer research.

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